Eur Food Res Technol (2008) 227:727–733
DOI 10.1007/s00217-007-0780-z

ORIGINAL PAPER

Enzymatic hydrolysis of steryl ferulates and steryl glycosides

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Received: 25 July 2007 / Revised: 2 October 2007 / Accepted: 10 October 2007 / Published online: 26 October 2007

Abstract Steryl ferulates (SF) and steryl glycosides (SG) are phytosterol conjugates found characteristically in cereals. Currently, little is known about their properties with respect to enzymatic hydrolysis. SF and SG were extracted and purified from rye and wheat bran. Their percentages of hydrolysis with different enzymes were studied using normal phase HPLC with UV detection for steryl ferulates and evaporative light scattering detection for steryl glycosides. Steryl ferulates were hydrolysed by mammalian digestive steryl esterases. It was further demonstrated that a mixture of steryl ferulates from rye and wheat was hydrolysed much more effectively than a steryl ferulate mixture from rice (commonly known as γ-oryzanol), suggesting greater bioavailability in non-rice steryl ferulates. Steryl glycosides were hydrolysed by a commercial microbial β-glucosidase preparation (cellobiase), but were not effectively hydrolysed by two other highly purified β-glucosidases. These results demonstrate for the first time the potential use of enzymes as a replacement for acid hydrolysis in analytical procedures for SG and also provide insights about the potential bioavailability of these sterol derivatives in human digestive systems.

Keywords Steryl ferulate · Steryl glycoside · Enzymatic hydrolysis · Plant sterol · γ-Oryzanol

Introduction

The increasing use of plant sterols in functional foods has also increased interest in natural sources of plant sterols such as seeds and cereal products. In addition to the free sterol alcohols and their fatty acid esters, cereals contain substantial amounts of glycosylated sterols or sterols esterified to phenolic acids (Fig. 1) [1].

In wheat and rye, the highest contents of steryl ferulates (SF, ferulic acid esters of sterols) are found in the bran. This demonstrates that steryl ferulates are localised in the outer layers of the cereal kernel. In wheat bran, for example, sterols as ferulic acid esters may contribute nearly 20% (30.8 mg/100 g d.w.) of total sterols [1]. Steryl glycosides (SG), on the other hand, may contribute 10% of the total plant sterols in cereals. The contents of sterols contributed by SG in wheat and rye grains are 7.5 mg/100 g d.w. and 8.3 mg/100 g, respectively [1]. These glycosylated sterols may be neglected in sterol analysis if the procedure is based on common chromatographic methods for cholesterol analysis that do not often include acid hydrolysis in sample preparation (as cholesterol does not often occur as glycosides). These steryl conjugates, which are characteristic to cereals, have been studied much less than the free sterols and steryl fatty acid esters, though they may confer some additional benefits with respect to the health effects of plant sterols. For example, steryl ferulates from rye and wheat inhibit lipid oxidation in moderate and high temperatures,

Electronic supplementary material The online version of this article (doi:10.1007/s00217-007-0780-z) contains supplementary material, which is available to authorized users.

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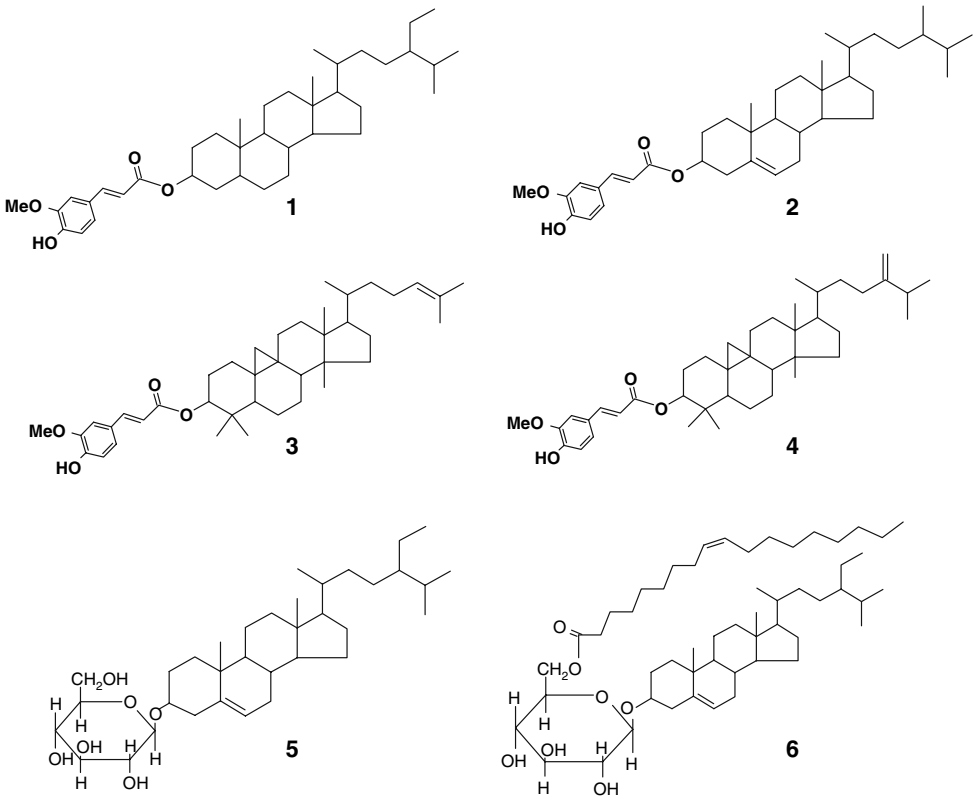

Fig. 1 Examples of phytosteryl ferulate and glycoside structures in cereals: sitostanyl ferulate **1**, campesteryl glucoside **2**, cycloartenyl ferulate **3**, 24-methylenecycloartanyl ferulate **4**, sitosteryl glucoside **5** and sitosteryl 6-oleoyl-*O*-glucoside **6**

and they have been shown to possess anti-inflammatory activity [2–4]. Steryl glycosides, on the other hand, have been reported to reduce benign prostate hyperplasia (enlargement of the prostate), and to have a number of other biological functions, such as anti-inflammatory, anti-diabetic, anti-ulcer and anti-cancer activities [5].

The metabolism of steryl ferulates and glycosides in humans is still poorly understood. It is thought that the steryl conjugates need to be hydrolysed to free sterols in order to inhibit cholesterol absorption [6, 7]. Except for steryl fatty acid esters, the activities of digestive enzymes like esterases and glycosidases towards phytosteryl conjugates have not been studied. Recent studies have shown that steryl ferulates from corn and rice are hydrolysed by steryl esterases, and that there are significant differences in the hydrolysis among different sterol moieties [6, 7]. This is of great significance, as the sterol composition among different cereal materials varies considerably. The mixture of SF from rice, called γ-oryzanol, contains principally 4,4′-dimethylsterols, i.e. sterols that have two methyl groups in position C-4 like cycloartenol (**3**) and 24-methylenecycloartanol (**4**). SF of wheat and rye, on the other hand, contain virtually only desmethylsterols that lack the methyl groups in C-4, such as sitosterol and campesterol (**2**) and their saturated counterparts sitostanol (**1**) and campestanol. Therefore, sterol composition of SF in a cereal may be a very important factor when estimating their effects as health promoting constituents. Like SF, also SG have been shown to inhibit cholesterol absorption from the intestine of rats [8], but absorption of SG itself is low [9]. Information on the metabolism of SG in humans or the hydrolysis of SG with mammalian digestive enzymes is scarce. Pegel [5] stated that the absorption of SG is only 20% of that of free sterols, thus resulting in about 1–2.5% absorption in animals.

In addition to an increased understanding of the metabolism of steryl conjugates more information on their enzymatic hydrolysis is needed to evaluate the possibilities of using enzymatic hydrolysis in the analysis of steryl conjugates. Common gas chromatographic methods for sterol analysis often include chemical hydrolysis (alkaline hydrolysis for SF and acidic hydrolysis for SG), after which sterols are analysed as free sterol alcohols. In addition to decreasing the use of chemicals, enzymatic hydrolysis would also serve as a more gentle method of hydrolysis. For example Δ5- and Δ7-avenasterols and citrostadienol are labile under acidic conditions and they may thus degrade in an analytical procedure including acid hydrolysis producing artefacts and resulting in lower than actual values. To our knowledge there are a few studies that have applied enzymatic hydrolysis of SG in the analysis, but none including enzymatic hydrolysis of SF. For example, Kesselmeier et al. [10] used β-glucosidase in the hydrolysis of SG from oat seeds. Some more recent studies, however,

have not been successful in similar hydrolysis. This may be due to various reasons, the most likely being that the SG hydrolysis demonstrated by Kesselmeier and co-workers was caused by activity of an unknown minor hydrolase in the crude enzyme preparation rather β-glucosidase. Thus with the modern highly purified enzyme preparations similar secondary activities are not present and SG is not hydrolysed.

In this study, we investigated enzymatic hydrolysis of steryl ferulates and steryl glycosides. Steryl ferulates were extracted from rye and wheat bran and their hydrolysis by mammalian steryl esterases was compared to that of a single compound (sitostanyl ferulate) and a mixture of steryl ferulates from rice bran with a different sterol composition (γ-oryzanol). Steryl glycosides from wheat and rye bran were hydrolysed with plant and microbial β-glucosidases that could be utilized inexpensively for routine analysis to liberate phytosterols from their conjugates before GC analysis and thus replacing acid hydrolysis.

Materials and methods

Materials

A steryl glycoside standard (>98%) was purchased from Matreya Inc. (Pleasant Gap, PA; USA). Sitostanyl ferulate was synthesised using the method of Condo et al. [11] and its purity was >99% using the HPLC method described in a later section. γ-Oryzanol was from CTS Organics (Atlanta, GA, USA). Sodium taurocholate was purchased from Fluka (Fluka Chimie GmbH, Buchs, Switzerland), Triton X-100, deoxycholic acid sodium salt and dimethylsulphoxide were from Sigma (St Louis, MO, USA). The enzymes used for the hydrolyses were: steryl esterase (EC 3.1.1.13) from bovine pancreas (Sigma C-3766, 211 units/g, 1 unit will hydrolyse 1 μmol of cholesteryl oleate per minute at pH 7.0 and 37 °C in the presence of taurocholate), steryl esterase (EC 3.1.1.13) from porcine pancreas (CEPM, 950 units/g, 1 unit hydrolyses 1 μmol of cholesterol ester per minute at 37 °C, pH 7.0, Worthington Biochemical Corporation, Lakewood, NJ, USA), microbial β-glucosidase (Megazyme E-BGLUC, 1 U/25 μl, 1 U of enzyme hydrolyses 1 μmol of *p*-nitrophenyl β-glucoside per minute at 40 °C and pH 4.0), β-glucosidase from almonds (BioChimica 49290, 7.55 U/mg, 1 U hydrolyses 1 μmol salicin [(2-hydroxymethyl)phenyl-β-D-glucoside] per minute at pH 5.0 and 35 °C, Fluka Chimie GmbH, Buchs, Switzerland) and microbial β-glucosidase (cellobiase from *Aspergillus niger*, ≥250 U/mg, Novozyme 188, Sigma C-6105). All solvents used were of HPLC grade and purchased from J.T Baker (Deventer, The Netherlands) or Rathburn Chemicals (Walkerburn, Scotland).

Preparation of cereal bran SF and SG substrates

Steryl ferulates and glycosides were extracted from wheat and rye bran (Fazer Company, Lahti, Finland) using the method previously reported [1]. Briefly, hot acetone was used to extract the lipids, which were then fractionated using solid phase extraction (SPE) with silica cartridges (BondElut, Silica, 500 mg, Varian Inc., USA). The SPE columns were with 2 × 2.5 ml of 1-heptane. Sterol classes were fractionated by adding 5 ml (2 × 2.5 ml) of each eluent and by collecting each eluting fraction. SF were first eluted with 1-heptane-diethyl ether (9:1, v/v) and 1-heptane-diethyl ether (1:1 v/v), and SG with acetone. Fractions containing SG were pooled and concentrated before use. The concentration of SG in the sample solution was determined using the normal phase-HPLC method for SG described below. The extract also contained minor amounts of acylated SGs and other polar lipids. The SPE-fractions containing steryl esters and free sterols were pooled and further purified using an acid–base wash procedure [12]. The resulting extract containing SF was further fractionated using preparative high performance liquid chromatography (prep-HPLC) with a silica column (Supelco PLC-Si, 250 × 21.2 mm, 12 μm, Bellafonte, PA, USA) and a mixture of heptane, isopropanol and acetic acid (99:1:0.1 v/v/v) as the mobile phase. UV-detector at a wavelength 315 nm was used to detect steryl ferulates and the corresponding fractions were collected, pooled and concentrated before use as a substrate. The concentration of SF in the sample solution was determined using the normal phase-HPLC method for steryl ferulates described below. Sterol compositions of the extracts were further verified with gas chromatography after saponification (SF), or acid hydrolysis and saponification (SG). After hydrolysis the released sterols were extracted and derivatised as trimethyl silyl (TMS)-ethers and analysed with gas chromatography with flame ionization detection, and quantified using dihydrocholesterol as an internal standard [13].

Enzymatic hydrolysis

Hydrolyses were performed using the procedure previously reported by Moreau et al. [7] with minor modifications. In brief, substrate stock solutions (0.005 mol/L) were prepared in either heptane or isopropanol. A mixture of SG (0.10 mg) was transferred into a test tube, evaporated to dryness with nitrogen stream and re-dissolved to 50 μl of dimethylsulphoxide (DMSO) before the enzyme was added in 500 μl of buffer solution (acetate buffer, pH 4.0, 0.05 mol/L or citrate buffer 0.1 mol/L, pH 5.0). For the hydrolysis of steryl ferulates 0.15 mg of substrate in solution was transferred into a test tube and evaporated to dryness with nitrogen stream. Sample was re-dissolved to 5 ml

of Trizma buffer (0.05 mol/L, pH 7.0) with 35 mmol/L of taurocholate as a detergent. Enzymes were added in buffer solution without detergent. Various amounts of enzymes were tested to see the effect of enzyme amount on the percent of hydrolysis. Steryl conjugates were hydrolysed for 0–18 h in a shaking water bath at 37 °C for SG and 40 °C for SF. After the incubation lipids were extracted with the Bligh–Dyer method [14] and the sterol conjugates were analyzed with NP-HPLC. Hydrolyses were generally performed generally twice with three replicates.

HPLC-method for the analysis of steryl conjugates

Steryl glycosides were analysed by normal phase (NP)-HPLC using a Hewlett-Packard 1100 liquid chromatograph (Agilent Technologies, Avondale, PA, USA) with a diol column (LiChrosorb Diol 5 µm, 100 × 3.0 mm, Chrompak, Raritan, NJC USA). Mobile phase was isocratic mixture of A:B 85:15 with A = hexane–acetic acid (1,000:1) and B = isopropanol and a flow rate of 0.5 ml/min and column heating at 30 °C. Steryl glycosides were detected with evaporative light scattering detector (Model 55, Richard Scientific, Novato, CA, USA) with detector temperature at 40 °C and nitrogen pressure at 2.0 bar, and quantified using a standard curve of 0.1–4 µg per injection of SG standard (from Matreya). Standard curve given as electronic supplemental material. Steryl ferulates were analysed with a Hewlett-Packard 1090 II liquid chromatograph (Waldbronn, Germany) with a diol column (LiChrosorb Diol 5 µm, 100 × 3.0 mm, VDS Optilab, Berlin, Germany) and an isocratic mobile phase of heptane, isopropanol and acetic acid (99:1:0.1 v/v/v) [3]. Detection was performed using a diode array detector at 315 nm and SF were quantified using a standard curve of 0.1–2.5 µg per injection of synthetic *trans*-sitostanyl ferulate [11] as an external standard. Standard curve given as electronic supplemental material. The percentage of hydrolysis was calculated as the decrease in the sum of *cis*- and *trans*-steryl ferulates.

Data analysis

Means of the hydrolysis percentages (min. three independent measurements) were compared using one-way Analysis of Variance and Tukey´s test at 95% confidence level.

Results and discussion

Sterol composition of steryl ferulate substrates

The sterol composition of the steryl ferulate extract obtained by prep-HPLC was analysed using gas chromatography after saponification to separate and quantify the individual sterol species. The mixture of the fractionated steryl ferulates from rye and wheat bran consisted of 46% campestanol, 32% sitostanol, 13% campesterol and 7% sitosterol. As expected from the literature the campestanyl and sitostanyl ferulates were the predominating species in this mixture [12, 15]. The proportions of different sterols were slightly different from the previously mentioned studies, which may be a result of the preparative-HPLC method, as the fraction collected did not necessarily contain all of the eluting steryl ferulates, but was rather collected from the middle of the eluting peak. The sterol composition of γ-oryzanol consisted of 42% 24-methylenecycloartanyl ferulate, 34% cycloartenyl ferulate, 18% campesteryl ferulate and 7% sitosteryl ferulate, which is similar to other reports [16, 17].

In the normal phase liquid chromatographic system (Diol-column and a mixture of heptane, isopropanol and acetic acid (99:1:0.1 v/v/v) as the eluent) all the steryl ferulate samples consisted of two peaks. We hypothesized that these are the *cis*- and *trans*-isomers of the ferulic acid moiety with retention times at 2.8 and 5.8 min, respectively (for chromatogram see Fig. 2a).

This is supported by the fact that both of these peaks had the spectrum of ferulic acid. Further, when the samples were analysed with reverse-phase-HPLC, based on separation caused by the different sterol moieties, no other peaks than steryl ferulates were detected. The predominant isomer of ferulic acid in steryl ferulates is *trans* (hence the identification of the larger peak as *trans*). Further, the relative retention times of *cis*- and *trans*-ferulates of dimethylsterols and desmethylsterols were in the same order as reported by Collins et al. [18]. The *cis-trans* configuration of ferulic acid may change when exposed to light during normal growing and harvesting. In addition to the natural variation, photoisomerisation may further occur during sample preparation and storage [19]. The original *cis-trans*-ratio of steryl ferulates was 0.51, 0.29 and 0.33 for sitostanyl ferulate, the mixture of rye and wheat steryl ferulates and oryzanol, respectively.

Hydrolysis of steryl ferulates

The steryl ferulates (sum of *cis*- and *trans*-isomers) showed significant differences in their percentages of hydrolysis by bovine and porcine steryl esterases (three units of enzyme per sample) (Table 1). The coefficients of variation for steryl ferulate contents were below 15% in the majority of cases, and below 25% in all. The most effective hydrolysis was obtained with the bovine steryl esterase using the SF mixture as substrate. After 4 h incubation 48% of steryl ferulates were hydrolysed. The percentage of hydrolysis increased even further to 66% when the incubation time was extended to 18 h. SF mixture was the best substrate

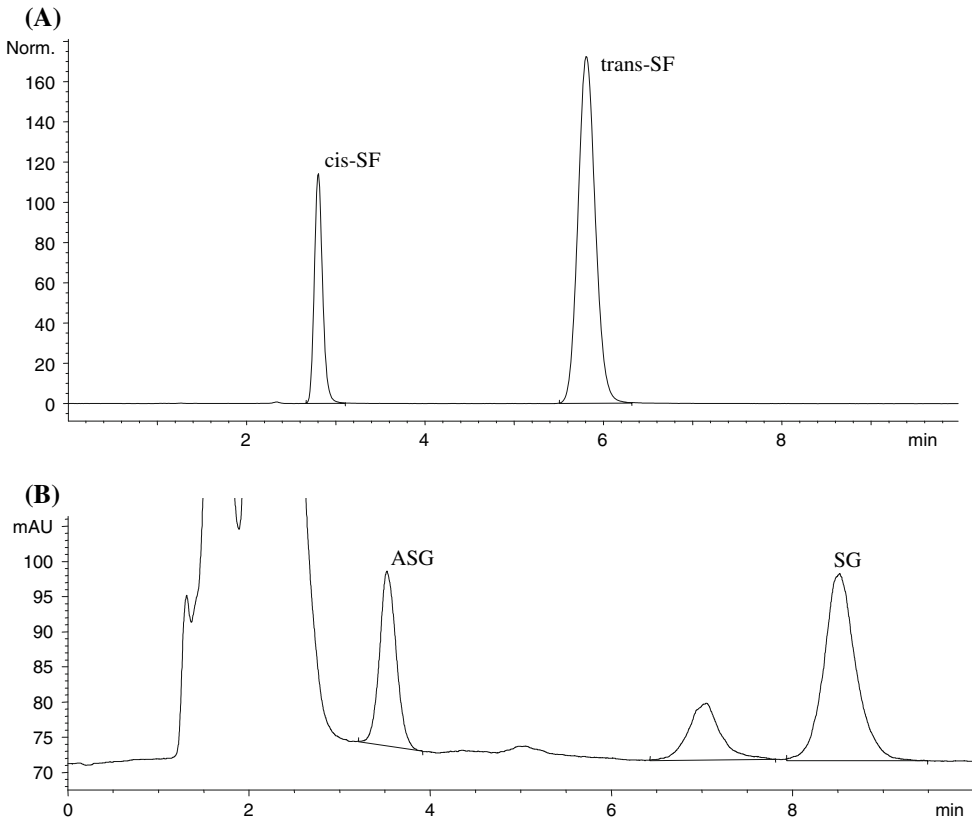

Fig. 2 Chromatograms of the analysis of steryl conjugates: **a** steryl ferulates (*SF*) using UV-detection at 315 nm, and **b** steryl glycosides (*SG*) and acylated steryl glycosides (*ASG*) using ELSD-detection

Table 1 Percent hydrolysis of total steryl ferulates (sum of *cis*- and *trans*-isomers) with bovine and porcine steryl esterases at 40 °C, for 18 h ($n = 6$)

Enzyme (3 U/sample)	Substrate (0.15 mg/sample)	% Hydrolysed 4 h	% Hydrolysed 18 h
Bovine steryl esterase	Sitostanyl ferulate	27[a]	28[a]
	SF mixture	48[b]	66[b]
	Oryzanol	5[c]	8[c]
Porcine steryl esterase	Sitostanyl ferulate	0[c]	7[c]
	SF mixture	25[ad]	58[b]
	Oryzanol	10[dc]	6[c]

Different superscript letters for the means within one time point denote a statistically significant difference

also for the porcine steryl esterase. The percentages of hydrolysis after 4 and 18 h incubation were 25 and 58%, respectively. The longer incubation time resulted in hydrolysis, which was comparable to that with the bovine steryl esterase.

Pure sitostanyl ferulate as individual substrate, on the other hand, was not hydrolysed as effectively as the SF mixture. With the bovine steryl esterase its percentage of hydrolysis was 27 and 28% for 4 and 18 h, respectively, which was significantly less than that of SF mixture, but yet higher than the hydrolysis percentage of γ-oryzanol. With the porcine steryl esterase the percentage of hydrolysis was very low, even after 18 h of incubation only 7% of the sitostanyl ferulate was hydrolysed. The previously reported hydrolysis percentage of sitostanyl ferulate with bovine steryl esterase after 4 h was much higher (85% hydrolysis) [7] than seen in this study. However, the experimental conditions were different (enzyme and taurocholate amounts) and thus the figures are not directly comparable.

Except for hydrolysis with porcine steryl esterase for 4 h, the percentage of hydrolysis for the mixture of steryl ferulates from rye and wheat (SF mixture) was significantly higher than that of γ-oryzanol. This indicates that the percentage of hydrolysis is indeed strongly dependent on the sterol composition of steryl ferulates. The hydrolysis percentage of γ-oryzanol was 10% or less with both enzymes and incubation times, which was significantly lower than the hydrolysis of the SF mixture. Thus it appears that ferulates of desmethylsterols are preferentially hydrolysed and are likely responsible for low-level the hydrolysis of steryl ferulates of γ-oryzanol. A similar phenomenon was reported by Miller et al. [6] who concluded that esters of dimethylsterols are not hydrolysed with steryl esterases. Further, hydrolysis percentage of γ-oryzanol was significantly lower than that of sitostanyl ferulate in a study by Moreau et al. [7] again supporting the view that desmethylsteryl ferulates are better substrates for mammalian steryl esterases than dimethylsteryl ferulates.

The hydrolysis percentage of SF was generally lower with porcine steryl esterase compared to the bovine enzyme. Only in the hydrolysis of γ-oryzanol were the hydrolysis percentages closely comparable. Similar results were reported by Miller et al. [6], in which the percentages of hydrolysis using the bovine steryl esterase were nearly twice the percentage obtained by the porcine enzyme. This study, together with the other reports with bovine steryl esterase [7] or with bovine, porcine and microbial steryl esterase [6] shows that the origin of the enzyme significantly affects the percentage of steryl ferulate hydrolysis, and that steryl ferulates are hydrolysed by the mammalian digestive enzymes. However, as hydrolysis is heavily dependent on the substrate sterol composition, these mammalian steryl esterases cannot be used as a replacement for chemical hydrolysis in the analytical procedure, which would require an enzyme with broad substrate specificity.

It is generally thought that in order for steryl ferulates to be biologically active (e.g. inhibit cholesterol absorption) they need to be hydrolysed in the gastrointestinal tract [6, 7]. Therefore, factors like sterol composition that affect hydrolysis are of great importance when evaluating the potential bioactivity of steryl ferulates from different sources. Rice and rice bran are considered excellent sources of γ-oryzanol, and nutraceutical preparations of steryl ferulates prepared from rice materials are available on the market. The content of γ-oryzanol has been reported to vary between 26 and 63 mg/100 g in European rice varieties and in Indian brown rice varieties between 50 and 72 mg/100 g [16, 20]. However, of this total amount only about 20–30% are desmethylsteryl ferulates and the rest dimethylsteryl ferulates that are not effectively hydrolysed by the mammalian steryl esterases. In other cereals like wheat, rye and corn all the steryl ferulates are desmethylsteryl ferulates, which are more effectively hydrolysed by digestive enzymes. Therefore, although the total steryl ferulate content in these cereals is lower than in rice, their sterol composition is comprised only of the potentially active desmethylsterols, thus increasing the importance of these cereals as sources of steryl ferulates. The content of desmethylsteryl ferulates in all these cereals (wheat, rye, corn and rice) is approximately the same [1, 12, 21]. Further, the steryl ferulates of rye and wheat were shown to inhibit lipid oxidation more effectively than γ-oryzanol [2], and furthermore desmethylsterols were shown to inhibit cholesterol absorption more effectively than dimethylsterols [22, 23]. Therefore it may be concluded that steryl ferulates from rye, wheat and corn are at least as effective and beneficial biologically as γ-oryzanol.

Hydrolysis of steryl glycosides

Three different β-glucosidases were studied for their capability to hydrolyse SG from wheat bran. This SG extract consisted of 66% sitosterol, 13% campesterol, 11% stanols and 10% other minor sterols. The first commercial enzyme, a β-glucosidase preparation from *A. niger* (also marketed as a cellobiase Novozyme 188, Sigma C-6105) effectively hydrolysed SG (Table 2). This enzyme was added at six different levels (5–90 U) and two different pH values (pH 4.0 and pH 5.0) using DMSO for dispersing the sample in buffer. At pH 4.0 the greatest percentage of hydrolysis (42.7%) was observed with the addition of 60 U of the enzyme. Somewhat higher percentage of hydrolysis was gained at pH 5.0 and 30 U of enzyme, which resulted in 50.3% hydrolysis of SG. The percentage of hydrolysis was further increased to 56.9% when sodium taurocholate (10 mmol) was used as a detergent in the system. Other detergents, namely deoxycholic acid (10 mmol) and Triton X-100 (0.4 mmol), appeared to inhibit of SG hydrolysis. The same β-glucosidase (cellobiase) preparation has been reported to hydrolyse isoflavone glycosides of a soy beverage to aglycones [24] and thus it is possible that the β-glucosidase enzyme preparation has a low substrate specificity enabling hydrolysis of other β-D glucosidic bonds than that of cellobiose. However, deglycosylation of isoflavones was not complete: of the original content of isoflavone glycosides 76% were hydrolysed by β-glucosidase enzyme. One reason for this may be product inhibition of the enzyme, which has been reported for cellobiases from *Trichoderma reesei*, *A. niger* and *Trichoderma viride* [25, 26]. Thus in order to obtain complete hydrolysis of SG with cellobiase, one might remove the evolving glucose from the system using for example another enzyme like glucose oxidase or hexokinase. These, as well as identifying the active enzyme in the cellobiase preparation should be studied in more depth in the future.

Two other commercial enzymes were also evaluated for their ability to hydrolyse SG. One was a highly purified microbial β-glucosidase from *A. niger* and the other was a

Table 2 Percent hydrolysis of steryl glycosides with β-glucosidase from *A. niger* (cellobiase Novozyme 188) at 37 °C, for 18 h

Enzyme addition (Units)	Dispersant/ detergent	% Hydrolysed (pH 4.0)	% Hydrolysed (pH 5.0)
5 U	DMSO	n.a.*	13.3
10 U	DMSO	n.a	32.4
20 U	DMSO	n.a.	37.2
30 U	DMSO	28.2	50.3
60 U	DMSO	42.7	37.6
90 U	DMSO	15.5	36.0
30 U	Taurocholate	n.a.	56.9
30 U	Deoxycholate	n.a.	19.9
30 U	Triton X-100	n.a.	16.6

* *n.a* not analysed

partially purified β-glucosidase from almonds. Both enzymes were evaluated at pH 5.0. Rather than hydrolysing SG, these enzymes increased the amount of SG after 18 h incubation. For example, with the specific microbial β-glucosidase hydrolysis of SG with 2 U of enzyme increased the amount of SG by 29% (data not shown), presumably due to hydrolysis of acylated steryl glycoside (ASG), which was also present in the sample. Furthermore, also the addition of 7 U of β-glucosidase from almonds increased the amount of SG by 13%. The hydrolysis procedure was conducted as described by Kesselmeier et al. [10], with the exception that a smaller amount of substrate and slightly higher incubation temperature were used in this study. Therefore, it may be assumed that the differences were caused by the enzyme preparation, rather than other differences in the procedure.

These results demonstrate the potential use of enzymes as a replacement for acid hydrolysis in analytical procedures for steryl glycosides. However, in order for the enzymatic hydrolysis to replace acid treatment in sterol analysis, the percentages of enzymatic hydrolysis of SG would need to be higher than those observed in Table 2. Because of these promising preliminary results, we feel that further studies will likely lead to the identification of optimal enzymatic conditions for hydrolysis of steryl glycosides. Nontheless, this study clearly demonstrates that SG can be enzymatically hydrolysed by microbial β-glucosidase product (cellobiase), but not the more highly purified β-glucosidase, thus suggesting that other enzymes or side activities in the enzyme product may also cause the hydrolysis. Furthermore, as no mammalian digestive enzymes were used in this study to hydrolyse steryl glycosides, these results do not directly demonstrate their possible hydrolysis in the human digestive system. It is possible that there may be some enzymatic hydrolysis of SG by endogenous enzymes from the plant material or microbial β-glucosidase from the intestinal microbiota, especially in the colon. However, to evaluate the possible significance of this for human nutrition much more information on metabolism of SG is needed.

Acknowledgments Laura Huikko, Tanja Nurmi and Mike Powell are thanked for technical assistance. The study was funded by the University of Helsinki and The Finnish Graduate School of Applied Bioscience (ABS). L. Nyström´s research grants from the University of Helsinki Jubilee Fund and Raisio Research Foundation are gratefully acknowledged. We would like to thank the Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, for hosting L. Nyström as a visiting scientist, during which time part of the studies herein reported were conducted.

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